Stem Cell Research 36 (2019) 101410

Contents lists available at ScienceDirect

Stem Cell Research

journal homepage: www.elsevier.com/locate/scr

Lab Resource: Multiple Cell Lines

Generation of two transgene-free human iPSC lines from CD133$^+$ cord blood cells

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A B S T R A C T

We have generated two human induced pluripotent stem cell (iPSC) lines from CD133$^+$ cells isolated from umbilical cord blood (CB) of a female child using non-integrative Sendai virus. Here we describe the complete characterization of these iPSC lines: PRYDi-CB5 and PRYDi-CB40.

Resource table.

Unique stem cell lines identifier	PRYDi-CB5 PRYDi-CB40
Alternative names of stem cell lines	CBiPS1sv-4F-5 CBiPS1sv-4F-40
Institution	Inbiomed Foundation (San Sebastian, Spain)
Contact information of distributor	A. Giorgetti, agiorgetti@cmrb.eu; X. Carvajal-Vergara, xcarvajal@unav.es
Type of cell lines	iPSCs
Origin	Human
Cell Source	Cord blood CD133$^+$ cells
Clonality	Clonal
Method of reprogramming	Sendai virus
Multiline rationale	isogenic clones
Gene modification	NO
Type of modification	N/A
Associated disease	N/A
Gene/locus	N/A
Method of modification	N/A
Name of transgene or resistance	N/A
Inducible/constitutive system	N/A
Date archived/stock date	March 2012
Cell line repository/bank	Cell lines registered at http://www.isciii.es/ISCIII/es/contenidos/fd-el-instituto/fd-organizacion/fd-estructura-directiva/fd-subdireccion-general-investigacion-terapia-celular-medicina-regenerativa/fd-centros-unidades/fd-banco-nacional-lineas-celulares/fd-lineas-celulares-disponibles/lineas-de-celulas-iPS.shtml
Ethical approval	Patient informed consent obtained by the Ethical Committee of Clinical Investigation of Donostia Hospital (Approval no. 9/05)

Resource utility

These human iPSC lines were derived from umbilical cord CD133$^+$ cells using Sendai virus that is a negative sense, single strand RNA virus that allows the generation of iPSCs without the transgene integration. These cells could be used as integration-free control iPSC lines.

Resource details

CD133 antigen is known as a stem cell marker for hematopoietic stem cells (HSCs) and progenitors, and is a valid substitute for CD34, commonly used for HSCs enrichment. Moreover, since CD133 is not expressed in late hematopoietic progenitors, this allows for the selection of a more homogeneous population enriched in HSC. Of note, CB CD133$^+$ cells express a subset of pluripotency-associated genes (*OCT4*,

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https://doi.org/10.1016/j.scr.2019.101410
Received 14 January 2019; Received in revised form 13 February 2019; Accepted 19 February 2019
Available online 28 February 2019

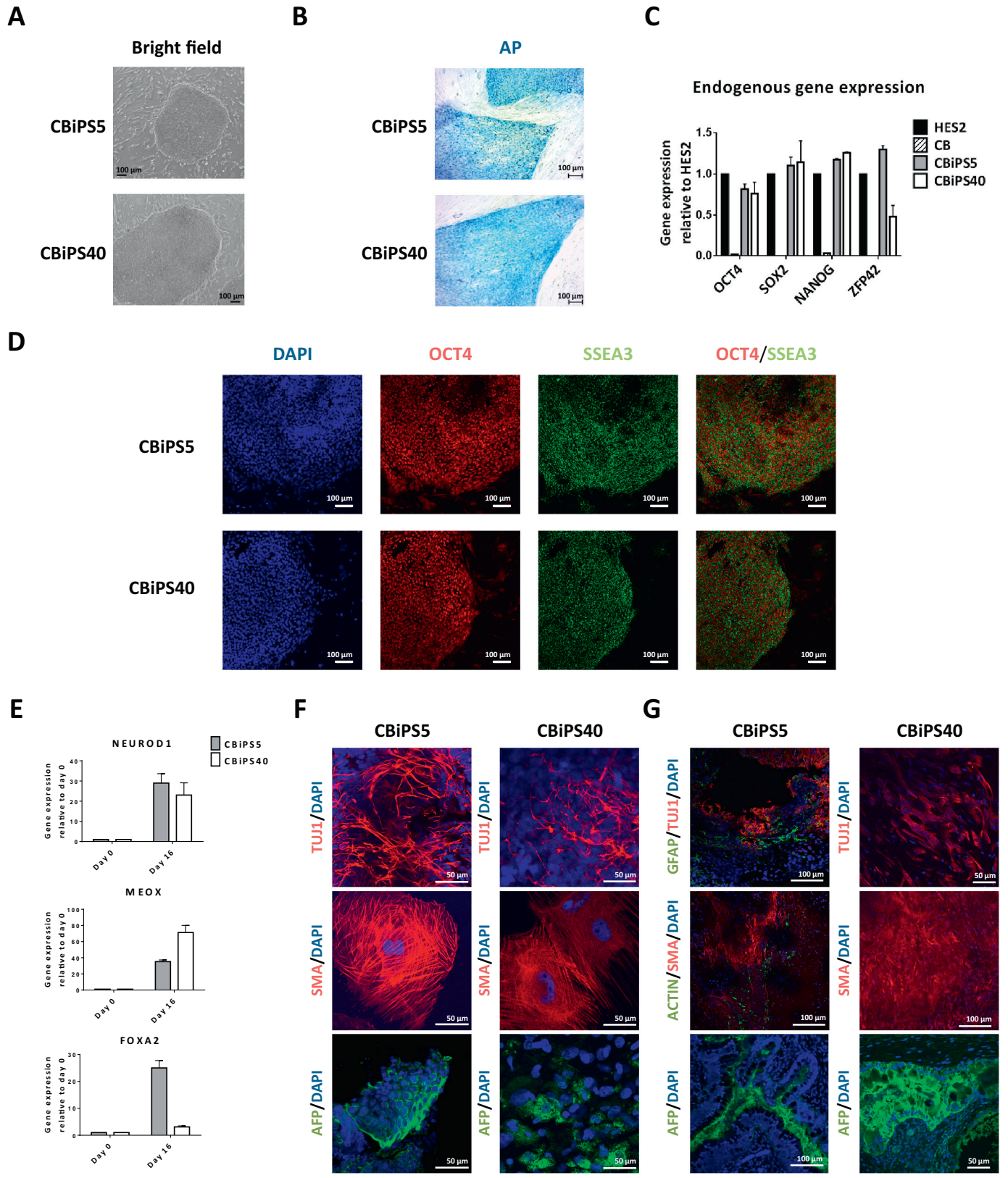

Fig. 1. Characterization of PRYDi-CB5 (CBiPS5) and PRYDi-CB40 (CBiPS40) human iPSC lines. A) A representative iPSC colony in bright field is shown. **B)** Analysis of the AP activity. **C)** Endogenous expression of pluripotency-associated genes (OCT4, SOX2, NANOG and ZFP42) by RT-qPCR. **D)** OCT4 and SSEA3 immunostaining. Nuclei: DAPI. **E)** Gene expression analysis by RT-qPCR of NEUROD3 (ectoderm), MEOX (mesoderm), and FOXA2 (endoderm) in undifferentiated (day 0) and differentiated iPSC (day 14). **F)** TUJ1 (ectoderm), SMA (mesoderm) and AFP (endoderm) protein expression analysis by immunostaining in undifferentiated (day 0) and differentiated iPSC (day 14). Nuclei: DAPI. **G)** Immunofluorescence analysis of TUJ1/GFAP (ectoderm), ACTIN/SMA (mesoderm) and AFP (endoderm) proteins in teratomas derived from the two iPSC lines. Nuclei: DAPI.

SOX2, *NANOG* and *CRIPTO*), albeit at much lower levels than human embryonic stem cells (HESCs) (Giorgetti et al., 2009). On the other hand, the endogenous levels of *cMYC* and *KLF4* are higher in CB CD133$^+$ cells compared with other adult somatic cells. The combination of low levels of pluripotency markers with the high levels of KLF4 and c-MYC allow enhanced reprogramming of CB CD133$^+$cells.

CD133$^+$ cells were isolated and infected with non-integrative Sendai virus vectors encoding *OCT4*, *SOX2*, *KLF4*, *c-MYC*. On day 3 after infection, cells were transferred onto plates containing a layer of irradiated mouse fibroblast feeders (MEFs) and cultured until human pluripotent stem cells-like colonies were observed, approximately 3 weeks post-infection. Colonies were manually picked for clonal expansion. Multiple iPSC clones were generated and here we show the characterization of two of these established iPSC lines, PRYDi-CB5 and PRYDi-CB40. The short tandem repeats (STR) analysis confirmed the genetic relatedness of both iPSC lines to their parental CB sample. These iPSC lines formed large and flat polygonal colonies of different shapes with smooth edges (Fig. 1A) and showed a high alkaline phosphatase (AP) activity (Fig. 1B).

Endogenous pluripotency-related transcription factors such *OCT4*, *NANOG*, *SOX2* and *ZFP42* were expressed (Fig. 1C). OCT4 transcription factor was expressed in the nucleus and SSEA3 pluripotency-related antigen was present in the surface of the iPSCs, assessed by immunofluorescence (Fig. 1D). Established cell lines were karyotypically normal (Sup Fig. 1A) and mycoplasma free (Sup. Fig. 1B). These iPSC lines have the potential to differentiate into cells of the three germ layers both *in vitro* and *in vivo*. Upon the *in vitro* differentiation of these iPSC lines, using an embryoid body (EB) differentiation assay (Carvajal-Vergara et al., 2010), ectoderm (NEUROD1 or TUJ1), mesoderm (MEOX or SMA) and endoderm (FOXA2 or AFP) markers were expressed, analyzed by RT-qPCR (Fig. 1E) or immunofluorescence staining (Fig. 1F), respectively. These cell lines were able to develop teratomas two months after their injection in NOD-SCID immunodeficient mice, and the immunohistological staining analysis confirmed that cells derived from the three germ layers such as TUJ1$^+$/GFAP$^+$ (ectoderm), ACTIN$^+$/SMA$^+$ (mesoderm) and AFP$^+$ (endoderm) were present (Fig. 1G).

Materials and methods

Generation of non-transgenic iPSCs

CB CD133$^+$ hematopoietic stem cells were isolated and cultivated as described in Giorgetti et al., 2009. One hundred thousand cells were transduced with CytoTune™-iPS 2.0 Sendai Reprogramming Kit (Invitrogen) at 37 °C for 24 h in CD133$^+$ growth medium containing 8 μg/mL polybrene. iPSCs were cultured on irradiated MEFs with KO-DMEM 20% KSR medium supplemented with Glutamax, penicillin/streptomycin, non-essential aminoacids (all from Gibco), b-mercaptoethanol (Sigma-Aldrich) and 20 ng/mL of bFGF (Peprotech) in an incubator with 5% CO_2 in air. The iPSC were passaged (1:6 ratio) every 5–6 days using 200 U/mL Collagenase IV (Gibco). After their characterization, these iPSC lines have also been adapted into feeder-free culture conditions at CIMA institute (Pamplona, Spain) and the iPSC lines can be grown on Corning Matrigel hESC-Qualified Matrix in mTeSR™1 medium (STEMCELL Technologies). We regularly harvest iPSCs every 4 days with TrypLE Express (Gibco) and seed approximately 300,000 cells per well of a 6-multiwell plate in mTeSR™1 supplemented with Y-27632 (STEMCELL Technologies) at 10 μM (Tables 1 and 2).

Cells were regularly tested for mycoplasma contamination by MycoAlert Mycoplasma Test (Lonza) and contaminations have not been detected.

STR analysis

To verify that human PRYDi-CB5 (CBiPS5) and PRYDi-CB40 (CBiPS40) iPSC lines derived from their parental CB sample, we performed STR analysis of three loci (D10S1214, D17S1290 and D7S796). The genomic DNA (gDNA) was isolated with Easy-DNA™ kit (Invitrogen). The PCR amplification was performed using a total of 50 ng of gDNA per reaction, the Taq polymerase with Standard Taq Buffer (New England Biolabs) and the primers listed in Table 3. The PCR conditions were as follows: 5 min at 94 °C, 35 x (5 min at 94 °C, 1 min at 55 °C and 1 min at 72 °C) and 7 min at 72 °C. A 2.5% agarose gel was used to analyze PCR products. A positive control, gDNA from the parental CB sample and a negative control, gDNA from BJ fibroblasts, was used.

Karyotyping

The iPSCs were grown on a T75 flask without feeders. Chromosomal analysis was performed in PRYDi-CB5 and PRYDi-CB40 at passage 8 and 10, respectively, using the standard GTG-banding method at the Genetics Service (Policlínica Gipuzkoa, San Sebastián). 15 metaphases per cell line were analyzed.

Immunofluorescence (IF) staining and alkaline phosphatase (AP)

The iPSCs or iPSC-derived differentiated cells were fixed with 4% paraformaldehyde for 15 min at room temperature. Pluripotency-associated (OCT4, NANOG, SOX2, SSEA3 and TRA-1-60) and differentiation (TUJ1, SMA, AFP) markers staining was performed as previously detailed (Carvajal-Vergara et al., 2010). Cells were counterstained with DAPI (Sigma) and analyzed with Zeiss LSM510-META confocal laser scanning microscope. AP Blue Membrane Substrate Solution was used following the manufacturer's instructions (Sigma-Aldrich).

Teramotas were fixed in 4% paraformaldehyde and embedded in paraffin. Slices of 5 um were obtained with a microtome. Antigen retrieval was performed with citrate buffer and nonspecific labelling was blocked in TBS 0.5% Triton blocking solution containing 3% donkey serum. Primary antibodies were diluted in blocking solution and incubated for 24 h at 4 °C. After washing in TBS the sections were incubated with their corresponding secondary antibody for 2 h at 37 °C. Finally, sections were washed, counterstained with DAPI and coverslipped with mounting medium.

All the primary and secondary antibodies used in this study are referenced in Table 3.

RT-qPCR

Total RNA was prepared with the RNeasy Mini Kit (QIAGEN) and reverse transcribed with Superscript III (ThermoFisher). Quantitative PCR (qPCR) analysis was performed in the StepOnePlus™ Real-Time PCR System using the power SyBR PCR green Master Mix. Sequences of pluripotency- and specific lineage-related markers primers are described in Table 3. The quantitative gene expression data were

Table 1
Summary of lines.

iPSC line names	Abbreviation in figures	Gender	Age	Ethnicity	Genotype of locus	Disease
PRYDi-CB5	CBiPS5	Female	Newborn	N/A	N/A	N/A
PRYDi-CB40	CBiPS40	Female	Newborn	N/A	N/A	N/A

Table 2
Characterization and validation.

Classification	Test	Result	Data
Morphology	Bright field	normal	Fig. 1A
Phenotype	AP	Activity shown	Fig. 1B
	RT-qPCR	Expression of endogenous pluripotency markers *OCT4*, *SOX2*, *NANOG*, *ZFP42*	Fig. 1C
	Immunofluorescence (IF) staining.	Positive staining for SSEA3 and OCT4	Fig. 1D
Genotype	Karyotype	46 XX Resolution: 400 bps	Sup. Fig.1A
Identity	STR analysis	3 loci tested, all matched	Available with the Authors
Mutation analysis (IF APPLICABLE)	Southern Blot OR WGS	N/A	N/A
Microbiology and virology	Mycoplasma	Mycoplasma testing by luminescence Negative	Sup. Fig.1B
Differentiation potential	Embryoid body formation	Expression of germ layer markers: Ectoderm: NEUROD3 (RT-qPCR); TUJ1 (IF) Mesoderm: MEOX (RT-qPCR); SMA (IF) Endoderm: FOXA2 (RT-qPCR); AFP (IF)	Fig. 1E Fig. 1F
	Teratoma formation	Expression of germ layer markers (IF): Ectoderm: GFAP, TUJ1 Mesoderm: ACTIN, SMA Endoderm: AFP	Fig. 1G
Donor screening (OPTIONAL)	HIV 1 + 2 Hepatitis B, Hepatitis C	N/A	N/A
Genotype additional info (OPTIONAL)	Blood group genotyping	N/A	N/A
	HLA tissue typing	N/A	N/A

Table 3
Reagents details.

Antibodies used for immunocytochemistry/flow-cytometry

	Antibody	Dilution	Company Cat # and RRID
Pluripotency markers	Rabbit anti-OCT4	1:200	BioVision Cat#3576-100 RRID#AB_2167563
	Rabbit anti-SOX2	1:75	Thermo Fisher Scientific Cat#PA1-16968 RRID#AB_2195781
	Goat anti-NANOG	1:50	Everest Biotech Cat#EB06860 RRID#AB_2150379
	Rat anti-SSEA3	1:4	Hyrbridoma Bank Cat#MC-631 RRID#AB_528476
	Mouse IgM anti-TRA-1-60	1:200	Thermo Fisher Scientific Cat#14-8863-80 RRID#AB_891612
Lineage-specific markers of the three germ layers	Rabbit anti-AFP	1:400	Agilent Cat#A0008 RRID#AB_2650473
	Rabbit anti-GFAP	1:500	Agilent Cat#Z0334 RRID#AB_10013382
	Mouse anti-TUJ1	1:500	Covance Research Products Inc. Cat#MMS-435P RRID#AB_2313773
	Mouse IgG2a anti-SMA	1:400	Sigma-Aldrich Cat#A5228 RRID#AB_262054
	Mouse IgM anti-ACTIN	1:400	Sigma-Aldrich Cat#A2172 RRID#AB_476695
Secondary antibodies	Alexa Fluor 546 Goat anti-Rabbit	1:1000	Thermo Fisher Scientific Cat#A-11035 RRID#AB_2534093
	Alexa Fluor 488 Donkey anti-Goat	1:500	Thermo Fisher Scientific Cat#A-11055 RRID#AB_142672
	Alexa Fluor 488 Goat anti-Rat	1:500	Thermo Fisher Scientific Cat#A-11006 RRID#AB_2534074
	Alexa Fluor 488 Goat anti-Mouse IgM	1:500	Thermo Fisher Scientific Cat#A-21042 RRID#AB_2535711
	Alexa Fluor 488 Donkey anti-Rabbit	1:500	Thermo Fisher Scientific Cat#A-21206 RRID#AB_2535792
	Cy™3 Donkey anti-Mouse	1:500	Thermo Fisher Scientific Cat#A-11035 RRID#AB_2534093

Primers

	Target (product size)	Forward/Reverse primer (5′-3′)
Pluripotency markers (qPCR)	*OCT4* (123 bp)	AACCTGGAGTTTGTGCCAGGGTTT/TGAACTTCACCTTCCCTCCAACCA
	SOX2 (150 bp)	AGAAGAGGAGAGAGAAAGAAAGGGAGAGA/GAGAGAGGCAAACTGGAATCAGGATCAAA
	NANOG (190 bp)	CCTGAAGACGTGTGAAGATGAG/GCTGATTAGGCTCCAACCATAC
	ZFP42 (267 bp)	AAAGCATCTCCTCATTCATGGT/TGGGCTTTCAGGTTATTTGACT
Differentiation markers (qPCR)	*NEUROD1* (200 bp)	CCCATGGTGGGTTGTCATATATTCATGT/CCAGCATCACATCTCAAACAGCAC
	MEOX (171 bp)	TGGAAGCGTGTGAAGGGAGGT/AAGGAAGAGGGTGAAGGTGGGATTG
	FOXA2 (107 bp)	GCATTCCCAATCTTGACACGGTGA/GCCCTTGCAGCCAGAATACACATT
House-Keeping Genes (qPCR)	*GAPDH* (87 bp)	TGCACCACCAACTGCTTAGC/GGCATGGACTGTGGTCATGAG
Identity (PCR)	D10S1214	ATTGCCCCAAAACTTTTTTG/TTGAAGACCAGTCTGGGAAG
	D17S1290	GCCAACAGAGCAAGACTGTC/GGAAACAGTTAAATGGCCAA
	D7S796	TTTTGGTATTGGCCATCCTA/GAAAGGAACAGAGAGACAGGG

normalized to expression levels of GAPDH housekeeping genes and error bars indicate ± s.d. of triplicates.

In vitro and in vivo differentiation

We used the *in vitro* differentiation procedure described in Carvajal-Vergara et al., 2010. Briefly, iPSC colonies detached with collagenase IV were disaggregated and dispersed into small clumps and cultured as EBs in iPSC medium without bFGF in ultra-low attachment plates (Corning Inc.). On day 1 the media was changed and EBs were cultured in StemPro34 medium supplemented with Glutamax, Pen/Strep (all from Gibco), 50 μg/mL ascorbic acid, 150 μg/mL transferrin and 4×10^{-4} 1-Thioglycerol (Sigma-Aldrich). On day 8 of differentiation, EBs were plated on 0.1% gelatin coated dishes and cultured in DMEM 10% FBS supplemented with Glutamax and Pen/Strep (all from Gibco) differentiation medium for 8 additional days before RNA collection for RT-

qPCR or cell fixation for immunostaining analyses.

To test the *in vivo* differentiation capability of iPSCs, one million cells were harvested with TrypLE, suspended in 100 ul of 50% (v/v) Matrigel (Corning) and 50% (v/v) PBS and injected intratesticularly in an 8-week old NOD-SCID mouse. Teratoma formation was observed after 2 months of infusion. The teratoma was sectioned and stained.

Acknowledgement

This work was supported by the “Ramón y Cajal” State Program, Ministry of Economy and Competitiveness (MINECO, RYC-2012-10981) to XCV; the “Retos de la Sociedad” State Program, MINECO SAF2016-79398-R (AEI/FEDER, UE) to XCV; and by the Basque Government, Department of Industry (SAIOTEK PE12IB003) and Obra Social Kutxa to AG.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scr.2019.101410.

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